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Sex-specific expression of a HOX gene associated with rapid morphological evolution

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Abstract

Animal diversity is shaped by the origin and diversification of new morphological structures. Many examples of evolutionary innovations are provided by male-specific traits involved in mating and sexual selection. The origin of new sex-specific characters requires the evolution of new regulatory interactions between sex-determining genes and genes that control spatial patterning and cell differentiation. Here, we show that sex-specific regulation of the HOX gene *Sex combs reduced* (*Scr*) is associated with the origin and evolution of the *Drosophila* sex comb — a novel and rapidly diversifying male-specific organ. In species that primitively lack sex combs, *Scr* expression shows little spatial modulation, whereas in species that have sex combs, *Scr* is upregulated in the presumptive sex comb region and is frequently sexually dimorphic. Phylogenetic analysis shows that sex-specific regulation of *Scr* has been gained and lost multiple times in *Drosophila* evolution and correlates with convergent origin of similar sex comb morphologies in several independent lineages. Some of these transitions occurred on microevolutionary timescales, indicating that HOX gene expression can evolve with surprising ease. This is the first example of a sex-specific regulation of a HOX gene contributing to the development and evolution of a secondary sexual trait.

Keywords: HOX genes; Evolution; *Drosophila*; Sexual differentiation; Gene regulation; Morphogenesis

Introduction

Male-specific morphological structures play a key role in sexual selection through male–male competition and male–female interactions. As a result, they provide some of the prime examples of evolutionary innovations and exaggerated characters, from the tails of birds of paradise to the horns of dung beetles (Diamond, 1986; Dominey et al., 1984; Eberhard, 2001; Emlen et al., 2005; Meyer et al., 1994; Moczek et al., 2006). The development of recently evolved male-specific organs offers a unique glimpse into the interplay between developmental pathways and selective pressures in shaping phenotypic diversity.

The origin of new morphological traits implies the origin of new molecular pathways to control their development. Research in a variety of models has shown that most evolutionary innovations are caused not by the appearance of new genes, but rather by the acquisition of new functions by pre-existing genes (Carroll, 2005; Carroll et al., 2001; Wilkins, 2002). Changes in gene regulation play a particularly prominent role in this process so that the origin of new developmental pathways and morphological structures often reflects the emergence of new regulatory interactions among previously unconnected genes. In the case of sexually dimorphic traits, genes responsible for spatial patterning and differentiation must be brought under the control of sex-determining genes (Kopp et al., 2000; Wilkins, 2002). In this report, we suggest that repeated gain and loss of sex-specific expression of the HOX gene *Sex combs reduced* (*Scr*) has contributed to the origin and diversification of the *Drosophila* sex comb.

The sex comb is a male-specific array of modified bristles that develops at a precise position on the prothoracic (T1) leg from a set of precursor bristles present in both sexes (Figs. 1A, B) (Hannah-Alavah, 1958; Tokunaga, 1962). Bristles on the ventral-anterior surface of the distal tibia and the proximal tarsal

Abbreviations: T1, T2, T3, pro-, meso-, and metathoracic legs; t1-t5, tarsal segments 1–5.

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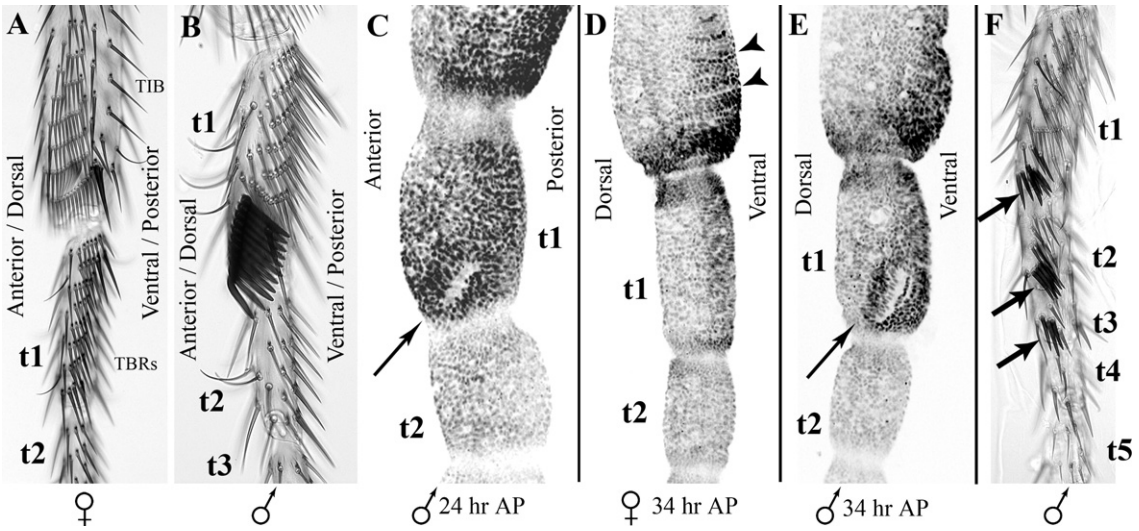

Fig. 1. Expression and function of *Scr* in *D. melanogaster*. Distal is down in all panels. TBR, transverse bristle rows; TIB, tibia; t1–t5, tarsal segments 1 through 5; hr AP, hours after pupariation. (A) Ventral view of the female T1 leg. Note the TBRs on the distal tibia and t1. (B) Anterior-ventral view of the male T1 leg. The sex comb develops from the most distal TBR on t1. (C) *Scr* expression in male T1 leg at 24 h AP, ventral view. Expression levels are similar in the distal tibia and t1, but much lower in t2. Arrow points to the developing sex comb. Bristle precursor cells that make up the sex comb do express *Scr*, but lie below the epithelium. (D) *Scr* expression in female T1 leg at 34 h AP, anterior view. Arrowheads point to the TBRs in the distal tibia. High *Scr* expression in the tarsus is confined to the ventral-anterior surface of t1. (E) *Scr* expression in male T1 leg at 34 h AP, anterior view. *Scr* expression is increased in distal t1 around the developing sex comb (arrow), whereas no such upregulation is observed in females (D). (F) T1 leg of a *tub*-Gal80^{ts}/UAS-*Scr*; *rn*-Gal4 male that was shifted from 18 to 29 °C at pupariation. Ectopic sex combs develop on the t2 and t3 segments (arrows). Note that the sex combs have failed to rotate and that the size of the t1 comb is reduced (compare to panel B).

segment of the T1 leg are arranged in tightly packed transverse bristle rows (TBRs) perpendicular to the proximo-distal axis of the leg. In *Drosophila melanogaster*, the sex comb develops from the most distal TBR on the first tarsal segment (t1). In males, this TBR rotates 90° so that the bristles become oriented from anterior/dorsal to posterior/ventral and point away from the leg (Fig. 1B). In addition, the bristles ("teeth") recruited into the sex comb undergo a number of morphological modifications. Sex comb teeth are curved and blunt rather than straight and pointed like other mechanosensory bristles, heavily melanized, and thicker than regular tarsal bristles. The sex comb is used by the male during courtship and mating to grasp the female's abdomen (Spieth, 1952), and some evidence suggests that sex comb morphology is subject to sexual selection (Markow et al., 1996; Polak et al., 2004).

The sex comb is a recent evolutionary innovation, and the majority of *Drosophila* species lack sex combs. It is present only in the *melanogaster* and *obscura* species groups of the subgenus *Sophophora* and (possibly independently) in the genus *Lordiphosa* (Hu and Toda, 2000; Lakovaara and Saura, 1982; Lemeunier et al., 1986). In the *melanogaster* species group, sex combs vary dramatically in size (number of teeth), position (either on t1 and t2, or only on t1), orientation (rotated or transverse, reflecting the presence or absence of bristle migration), and the size, shape, and color of teeth (Kopp and True, 2002). In all species that have rotated sex combs, individual teeth are enlarged, curved, and melanized, while in species with transverse sex combs the teeth do not differ greatly from regular bristles (Fig. 2). In species that primitively lack sex combs or have lost them secondarily, bristle pattern on the T1 leg is identical in males and females, and the female pattern is in fact conserved throughout Drosophilidae.

In this report, we reconstruct the evolution of sex comb morphology and *Scr* regulation. We find convincing evidence that sex-specific expression of *Scr* has been gained and lost multiple times in *Drosophila* evolution, with some of these changes occurring on microevolutionary timescales. In each independent lineage, increased *Scr* expression in males is associated with the presence of a large, rotated sex comb. Experimental analysis in *D. melanogaster* confirms that *Scr* determines sex comb position along the proximo-distal leg axis. These observations suggest that repeated gain and loss of sex-specific *Scr* expression have played an important role in sex comb origin and diversification.

Materials and methods

Ectopic Scr expression

Ectopic expression of *Scr* during larval development causes deletion and fusion of tarsal segments (Barmina et al., 2005; Shroff et al., 2007). To circumvent this problem, we used the temperature-sensitive Gal80 repressor of the Gal4 transcription factor (McGuire et al., 2004). Gene expression was driven by the *rn*-Gal4 enhancer trap, which is expressed uniformly from distal t1 to distal t4. *tub*-Gal80^{ts}/UAS-*Scr*; *rn*-Gal4 flies were raised at the permissive temperature (18 °C) until pupariation and shifted to the restrictive temperature (29 °C) as white prepupae (0–1 h after pupariation). Under this regime, tarsus growth occurred normally, although joint formation was sometimes incomplete. The ability of *Scr* to induce sex comb development in females and outside of the anterior-ventral leg surface was tested in UAS-Gal4/UAS-*Scr*; *rn*-Gal4 flies at 25 °C.

Antibody staining and image analysis

To obtain precisely synchronized cohorts of pupae, white prepupae were collected from culture bottles, sexed, placed on a moist Kimwipe in a Petri dish, and aged at 25 °C in 70% humidity. Once aged, the pupae were attached to

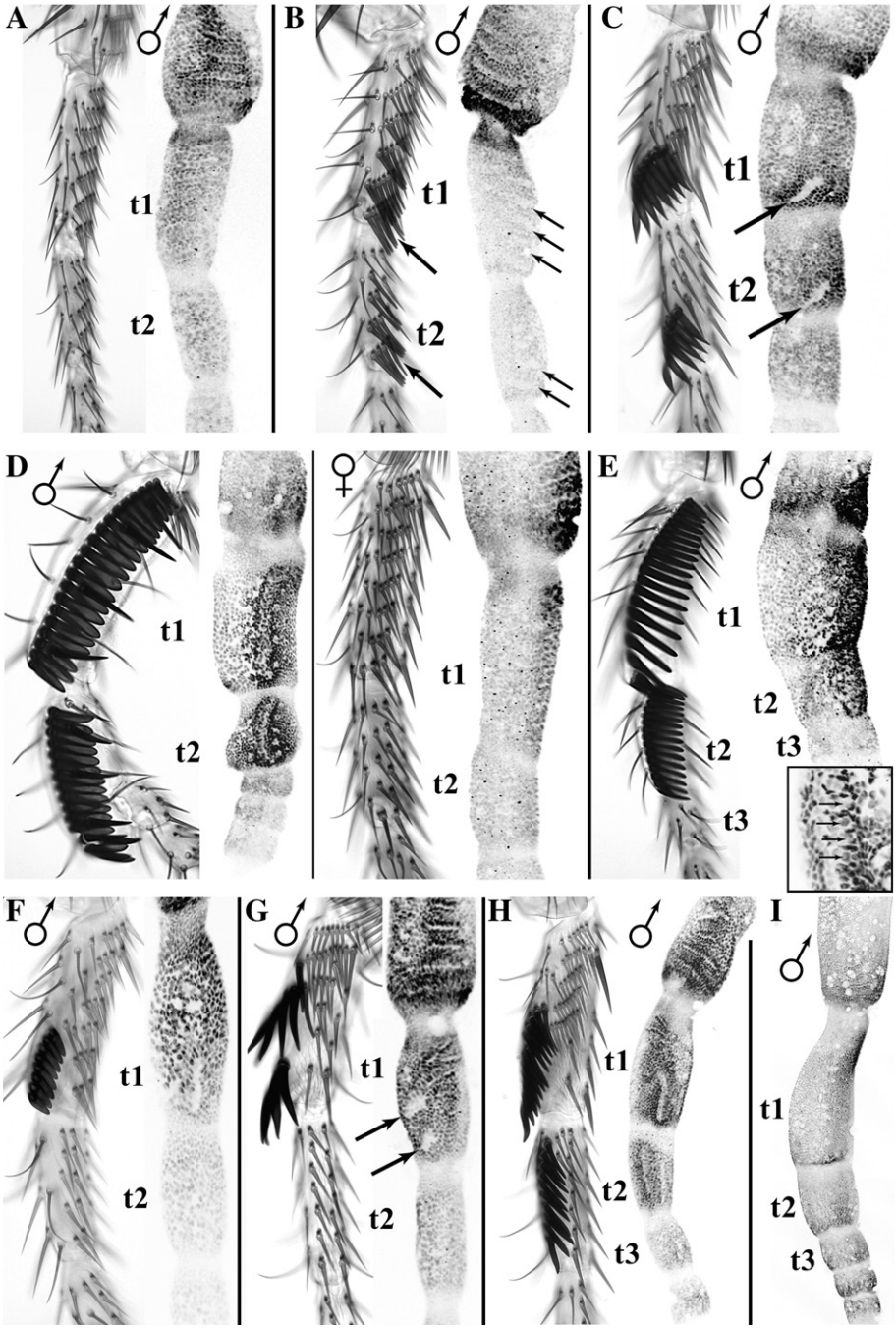

Fig. 2. *Scr* expression in *Drosophila* species with different sex comb morphologies. Distal is down and anterior is to the left in all panels. See Fig. 4 and Supplementary Fig. 1 for phylogenetic relationships among these and other species. Bristle cells that make up the sex comb do express *Scr* but lie below the epithelium (see inset in panel E). (A) Male bristle pattern and pupal *Scr* expression in *D. willistoni*, a species that primitively lacks sex combs. *Scr* expression is only weakly modulated in the tarsus and is lower in t1 than in the distal tibia. (B) Male bristle pattern and pupal *Scr* expression in *D. ananassae*, a species with a transverse, weakly modified sex comb (thick arrows). Thin arrows in the right panel point to the developing sex comb. (C) *D. pseudoobscura* male. The presence of sex combs on t1 and t2 (arrows) correlates with increased *Scr* expression in both of these segments, comparable to the expression in the distal tibia. (D) Female and male of *D. ficusphila*. Note the differences in *Scr* expression in t1 and t2 between male and female. (E) Male of *D. kikkawai*. *Scr* expression levels are similar in t1 and t2, correlating with the presence of sex combs on both segments, but are much lower in the rest of the tarsus. Inset shows *Scr* expression in bristle precursor cells (arrows) at a later stage, when they become polyploid. (F) Male of *D. nikananu*, a species that has secondarily lost the t2 sex comb. (G) Male of *D. biarmipes*. *Scr* expression is higher in t1 than in t2, correlating with the presence of sex combs only on t1. (H) Male bristle pattern and pupal *Scr* expression in *KB866*, an undescribed species. Note the presence of sex combs and increased *Scr* expression in t1 and t2. (I) *D. prolongata*, a close relative of *KB866* that has secondarily lost all sex combs and TBRs. *Scr* expression in this species is sexually monomorphic and is uniformly low throughout the tarsus.

sticky tape and cut with a razor blade into ventral and dorsal halves. The ventral halves were removed from the pupal case and fixed in 4% formaldehyde (EM grade, Polysciences) in fixation buffer (0.1 M PIPES pH 6.9, 1 mM EGTA pH 7.0, 2 mM $MgSO_4$, 1% Triton X-100) for 30 min at room temperature. Samples were washed and transferred to a depression glass filled with wash buffer (50 mM Tris–HCl pH 6.8, 150 mM NaCl, 0.5% NP40, 1 mg/ml BSA). Pupal

cuticle was ruptured at the base of each leg using forceps, and the leg was severed near the distal femur/proximal tibia boundary and pulled out of its pocket of pupal cuticle. Dissected legs were washed twice for 10 min each in the wash buffer, blocked overnight at 4 °C in wash buffer supplemented with 5 mg/ml BSA and incubated with the primary antibody overnight at 4 °C. Samples were then washed several times in the wash buffer and incubated for 2 h with secondary antibodies at 1:200 dilution, washed several times, and mounted in VectaShield (Vector Labs). The primary antibodies were: mouse α-Scr 6H4.1 (supernatant, 1:10) (Glicksman and Brower, 1988), mouse α-Dac Mabdac1-1 (supernatant, 1:10) (Mardon et al., 1994), and rat α-Bab2 (polyclonal serum, 1:2000) (Couderc et al., 2002). Confocal imaging was performed on an Olympus FV1000 laser scanning microscope.

Drosophila species used in this study differ in the duration of pupal development. However, comparable stages of leg development can be identified by examining the alignment of TBR bristles in the distal tibia. In most species, gene expression was examined at multiple time points between 20 and 45 h after pupariation (AP).

Technical variation in signal intensity prevents direct quantitative comparison of *Scr* expression levels between species. Instead, we examined the spatial pattern of *Scr* within each leg specimen in males and females of different species. In particular, we noted the differences in expression between the distal tibia, t1, and the more distal tarsal segments, as well as the differences between anterior-ventral and other leg surfaces. We used these observations to determine whether *Scr* expression in each species was upregulated in t1 and/or t2, and whether it was sexually dimorphic or monomorphic. The results are recorded in Fig. 4.

Quantitative rt-PCR

Sexed pupae were synchronized and aged as described above. Analysis was performed at 24 h AP in *D. willistoni* and at 29 h AP in *D. ficusphila*. Once aged, the pupae were attached to sticky tape, removed intact from the pupal cases, and placed in a depression glass filled with Trizol (Invitrogen), which was kept on ice thereafter. The front and middle legs were removed under Trizol using sharp forceps and placed in tubes containing Trizol. The dissected portion of the leg included only the tarsal segments and distal tibia. Total RNA was then isolated and double-stranded cDNA template synthesized as described (Barmina et al., 2005). In each species, we compared *Scr* expression between first and second legs of males and females using 3–4 independent replicates composed of 30–35 individuals each.

Part of the second *Scr* exon was amplified and sequenced from *D. ficusphila* using primers CGAGATGCACTCGCTTCATCCASGGA and CGTACCAGTTYGTCAA-CTCGCTGGCCTC; published genome sequence was used for *D. willistoni*. Primers for quantitative rt-PCR (qPCR) were designed using TaqMan probe and primer design software (Applied Biosystems) based on species-specific sequences and were GACCCCTGGCTTTAAAATGGA and GGCCAGCGAGTTGACGAA for *D. ficusphila* and TTTGCCATTGCCGCTATTCT and GCGGTGATTCGGATTCAGA for *D. willistoni*. qPCR reactions were performed using 15 ng of cDNA template, 0.2 μM of each primer, and SybrGreen PCR master mix on an ABI-7900 real-time thermal cycler. Gene expression levels were quantified as cycle thresholds (CT) — the number of cycles required to reach exponential amplification. The same procedures were used to quantify the expression of *actin5C* in each replicate sample to account for possible variation in the starting amount of RNA and the efficiency of reverse transcription among samples. Normalized *Scr* expression levels were measured as $CT_{Scr}/CT_{actin5C}$.

Since different *Scr* and *actin5C* qPCR primers were used in each species, gene expression levels cannot be compared directly between species. Instead, we used a two-tailed *t*-test to test whether the normalized *Scr* expression levels differed significantly between male and female T1 legs in each of the four species. *P* values were considered significant if they fell below the Bonferroni-corrected threshold of 0.025 (0.05/2).

CT value is a negative logarithmic function of transcript abundance: higher initial concentration allows PCR to reach exponential stage after fewer cycles. To translate differences in CT values into relative transcript abundance, we calibrated qPCR measurements using serial dilutions of plasmid DNA as described (Barmina et al., 2005). The ratio of transcript abundance in two samples (A and B) was calculated from mean CT values as $2.853^{CT(Scr)A-CT(Scr)B}/2.853^{CT(Act5c)A-CT(Act5c)B}$. Although these ratios are of course approximate, they allow us to compare the extent of sexual dimorphism between species.

Phylogeny reconstruction

Data collection and sequence alignment were carried out as described (Kopp, 2006). Phylogeny reconstruction was performed using a concatenated alignment of up to 9756 bases (from 5 to 14 different nuclear and mitochondrial loci) per species. Genbank accession numbers for sequences used in this study are listed in Supplementary Table 1. Bayesian phylogenetic analysis was performed on the concatenated data set using *MrBayes v3.0* (Huelsenbeck and Ronquist, 2001). Substitution model parameters were estimated as part of the analysis, starting from default priors. Each locus was treated as a separate partition that followed a full GTR+I+Γ model with six substitution rates. Each locus was allowed to have different substitution model parameters, but all partitions were constrained to the same tree topology and the same set of branch lengths. Analysis was repeated 4 times starting with random trees. Each time it was run for 5,000,000–10,000,000 generations, with the first 300,000 generations discarded, and the trees were sampled every 10,000 generations. Each time the analysis produced identical tree topology and similar partition probabilities (Supplementary Fig. 1). Reconstruction of ancestral character states on this phylogeny is described in the Supplementary data.

Results

Divergent and convergent evolution of sex comb morphology

To understand how the remarkable diversity of sex comb structures arose, we reconstructed phylogenetic relationships among 46 species of the *melanogaster* and *obscura* species groups using combined DNA sequences of 12 nuclear and 2 mitochondrial loci (Supplementary Fig. 1). Based on this strongly supported phylogeny, we analyzed the evolution of sex comb morphology using Bayesian reconstruction of ancestral character states (Pagel et al., 2004) (Supplementary Fig. 2). For each *Drosophila* clade, we estimated the probability that the latest common ancestor of that clade had (1) a rotated sex comb and (2) a sex comb on the second tarsal segment. We found that the latest common ancestor of the *melanogaster* and *obscura* species groups had sex combs on both t1 and t2 (>99% probability) and that the t2 comb was lost independently in five separate lineages: *D. tolteca*, *D. biarmipes, D. eugracilis, D. nikananu*, and the *melanogaster* subgroup (Supplement Fig. 2). Evolutionary transitions between rotated and transverse sex combs have been so frequent that ancestral states could not be reconstructed with confidence (Supplementary Fig. 2). However, using maximum likelihood and Bayesian statistical tests, we were able to reject the hypothesis that rotated sex combs evolved through a single gain followed by multiple losses, as well as the hypothesis that they evolved through multiple independent gains with no losses (Table 1). These hypotheses were rejected for the *melanogaster* species group as a whole, as well as for the smaller "Oriental" sub-clade (Table 1 and Supplementary Fig. 1). We conclude that rotated sex combs composed of strongly modified teeth have been gained and lost multiple times, and there is strong evidence that similar sex comb morphologies have evolved convergently in several independent lineages (Supplementary Fig. 2).

Table 1
Bayes factors in support of multiple gains and losses of sex comb rotation and sex-specific *Scr* expression

	Sex comb rotation	
	H_0: single gain and multiple losses (latest common ancestor had a rotated sex comb, and there have been no subsequent gains of rotated sex comb)	H_0: multiple gains with no losses (latest common ancestor did not have a rotated sex comb, and there have been no subsequent losses of rotated sex comb)
	H_1: multiple gains and losses (latest common ancestor had a rotated sex comb, and there have been both gains and losses of rotated sex comb subsequently)	H_1: multiple gains and losses (latest common ancestor did not have a rotated sex comb, and there have been both gains and losses of rotated sex comb subsequently)
melanogaster species group	7.17	4.33
"Oriental" clade	5.26	4.42
	Sex-specific *Scr* expression	
	H_0: single gain and multiple losses (latest common ancestor had sexually dimorphic *Scr* expression, and there have been no subsequent gains of dimorphic expression)	H_0: multiple gains with no losses (latest common ancestor did not have sexually dimorphic *Scr* expression, and there have been no subsequent losses dimorphic expression)
	H_1: multiple gains and losses (latest common ancestor had sexually dimorphic *Scr* expression, and there have been both gains and losses of dimorphic expression subsequently)	H_1: multiple gains and losses (latest common ancestor did not have sexually dimorphic *Scr* expression, and there have been both gains and losses of dimorphic expression subsequently)
melanogaster species group	5.62	4.02
"Oriental" clade	4.18	3.55

See Supplementary information for details of character reconstruction and hypothesis testing.

Scr controls sex comb position

Sex comb development in the T1 leg of *D. melanogaster* depends on the HOX gene *Sex combs reduced* (*Scr*). Sex comb is lost entirely in homozygous *Scr* null clones (Struhl, 1982), while a strong reduction in the number of sex comb teeth is seen in hypomorphic mutants and in flies heterozygous for *Scr* nulls or deficiencies (Kaufman et al., 1980; Lewis et al., 1980; Pattatucci et al., 1991). Conversely, ectopic *Scr* expression in T2 and T3 legs is sufficient to induce ectopic sex combs in these legs (Hannah-Alava, 1964; Kaufman et al., 1980; Shroff et al., 2007).

During early pupal development, when the sex comb and other leg bristles are specified, *Scr* levels are high in the presumptive TBR region on the ventral-anterior surface of the distal tibia and t1 and much lower in other parts of the T1 leg (Shroff et al., 2007) (Figs. 1C–E). Thus, *Scr* is only expressed at a high level in those parts of the T1 leg that produce T1-specific morphological structures. Moreover, we find that *Scr* expression in *D. melanogaster* is sexually dimorphic: in males, *Scr* levels are highest at the distal end of the t1 segment around the developing sex comb, whereas no such upregulation is observed in females (Figs. 1D, E).

In *D. melanogaster*, the sex comb is restricted to the first tarsal segment, and so is high *Scr* expression (Fig. 1). In other species, however, sex combs are also present on the second tarsal segment (Supplementary Fig. 2). In all cases, the presence of sex combs on the t2 correlates with distal expansion of *Scr* expression (Fig. 2), suggesting that spatial modulation of *Scr* determines the proximo-distal position of the sex comb. To test this possibility, we expressed *Scr* ectopically in distal tarsal segments during early pupal development. Ectopic sex combs were observed in t2 and t3 (Fig. 1F), indicating that high levels of *Scr* are sufficient to induce sex comb development in these segments. Interestingly, both the normal sex comb on t1 and the ectopic sex combs on t2 and t3 fail to rotate in these mutants, suggesting that a boundary between high and low *Scr* expression is necessary for initiating or guiding bristle migration.

Even very high *Scr* expression cannot induce sex combs in females, suggesting that other genes expressed in a sex-specific manner are required in parallel with *Scr*. Similarly, sex combs are only induced by ectopic *Scr* on the anterior-ventral leg surface and only in the distal portion of each tarsal segment (Fig. 1F), indicating that the regulatory inputs of *Scr* and the sex determination pathway are superimposed on an underlying spatial pre-pattern. Consistent with these observations, sex combs develop from only a subset of cells that upregulate *Scr* at the pupal stage (Fig. 2). Thus, high levels of *Scr* are necessary, but not sufficient for sex comb development.

Species- and sex-specific Scr expression reflects sex comb diversity

To understand the role of *Scr* in the origin and evolution of sex combs, we mapped *Scr* expression and sex comb morphology onto the molecular phylogeny of the *melanogaster* and *obscura* species groups (Fig. 3 and Supplementary Figs. 1 and 2). We found a strong correlation between *Scr* expression and the presence, size, and morphology of the sex comb. In outgroup species that primitively lack sex combs (*D. virilis*, *D. hydei*, and *D. willistoni*), *Scr* expression shows only weak spatial modulation in pupal T1 legs and does not differ visibly between males and females (Fig. 2A). In species that have transverse sex combs composed of weakly modified bristles (e.g., *D. ananassae*, *D. takahashii*, *D. elegans*, and *D.*

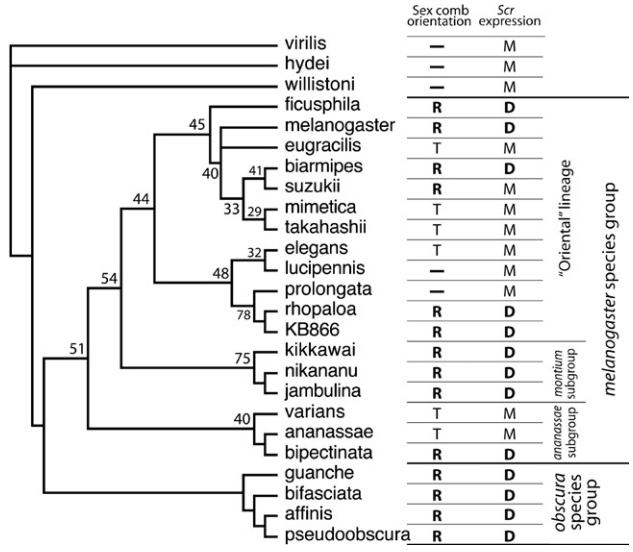

Fig. 3. Phylogenetic distribution of rotated sex combs and sex-specific *Scr* expression. See Supplementary Figs. 1 and 2 for a larger phylogeny. R, rotated sex comb composed of strongly modified teeth; T, transverse sex comb composed of weekly modified bristles; "–", sex comb absent; D, sexually dimorphic *Scr* expression; M, monomorphic *Scr* expression. All species with transverse sex combs show sexually monomorphic expression, while most species with rotated sex combs show dimorphic *Scr* expression. The only exception to this pattern is *D. suzukii*, which has a small sex comb that rotates only partially. Numbers at each internal node indicate the probabilities that the latest common ancestor of that clade had a rotated sex comb. These values do not deviate greatly from 50%, and neither hypothesis is significantly more likely than the other at any of the nodes (see Supplementary information for details on character reconstruction and hypothesis testing).

eugracilis), and/or have secondarily lost sex combs (*D. lucipennis* and *D. prolongata*), *Scr* expression is lower in the tarsus than in the distal tibia and does not show obvious upregulation in the presumptive sex comb region (Fig. 2B). However, in species that have rotated sex combs composed of strongly modified teeth (e.g., *D. pseudoobscura*, *D. biarmipes*, *D. ficusphila*, and *D. jambulina*), *Scr* expression is higher in the presumptive TBR and sex comb region than in the rest of the tarsus (Figs. 2C–G).

The proximo-distal extent of high *Scr* expression invariably corresponds to sex comb position: in species that have sex combs on both t1 and t2, *Scr* expression is upregulated in both these segments (Figs. 2C–E), whereas in species that have sex combs only on t1 *Scr* is also upregulated only in t1 (Figs. 1C, 2F, G). The presence of sex combs on t2 is the ancestral condition in the *melanogaster* species group, and the t2 comb was lost independently in several lineages (see above). In all such cases, we observe a concomitant reduction of *Scr* expression in the t2. Proximo-distal patterning genes such as *dachshund* and *bric a brac* (Docquier et al., 1997; Dong et al., 2001; Godt et al., 1993) are expressed identically in species with or without t2 sex combs, arguing against a general transformation of t2 to t1 identity in species with distal sex combs (Fig. 4).

In species that have rotated sex combs composed of strongly modified teeth, both the spatial extent and the cell-by-cell levels of *Scr* expression are greater in males than in females (Figs. 2D, E and 3). On the other hand, species that have no sex comb or a transverse sex comb composed of weakly modified bristles show no detectable sexual dimorphism in *Scr* expression. This correlation is observed throughout the *melanogaster* and *obscura* species groups (Fig. 3). To confirm the results of antibody staining, we quantified *Scr* mRNA levels in early pupal legs by quantitative rt-PCR. In *D. willistoni*, which primitively lacks sex combs, *Scr* transcript levels do not differ between male and female T1 legs ($P=0.53$; Fig. 5A). On the other hand, in *D. ficusphila* *Scr* expression is 7-fold higher in males than in females in the T1 leg ($P=0.0003$; Fig. 5B), but does not differ in the T2 legs ($P=0.82$). In *D. ficusphila*, Scr transcript levels are 26-fold higher in the T1 than in the T2 legs in males, but only 4-fold higher in females, whereas in *D. willistoni* the difference between T1 and T2 is 9-fold in both sexes.

Repeated gain and loss of sex-specific Scr expression

We reconstructed the evolution of sex-specific *Scr* expression in our phylogenetic framework using Bayesian estimation of

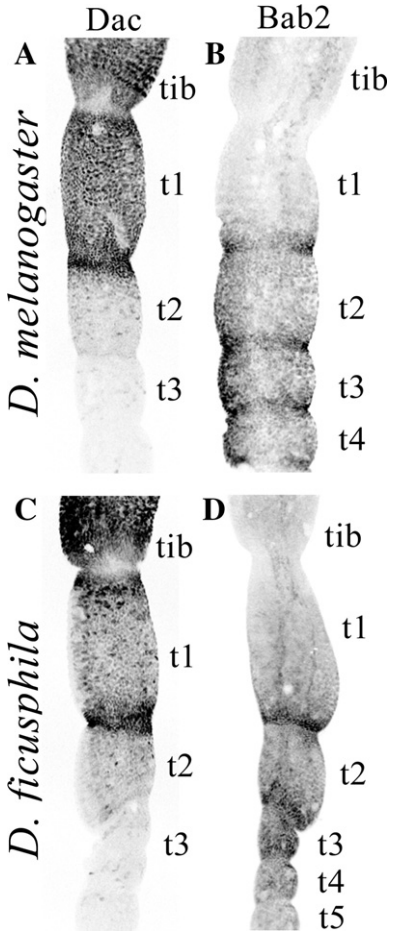

Fig. 4. Dac and Bab2 expression in species with and without t2 sex combs. In both *D. melanogaster* and *D. ficusphila*, Dac expression at the pupal stage is high in the tibia and t1, low in t2, and absent in the more distal segments (A, C). Also in both species, Bab2 expression is higher in t2–t4 than in distal t1 and is absent in proximal t1 and tibia (B, D). Bab2 expression is also increased in the distal part of each segment relative to the proximal part of the same segment. In these and other species, Dac and Bab2 are coexpressed in distal t1 and t2, regardless of the proximo-distal position of the sex comb.

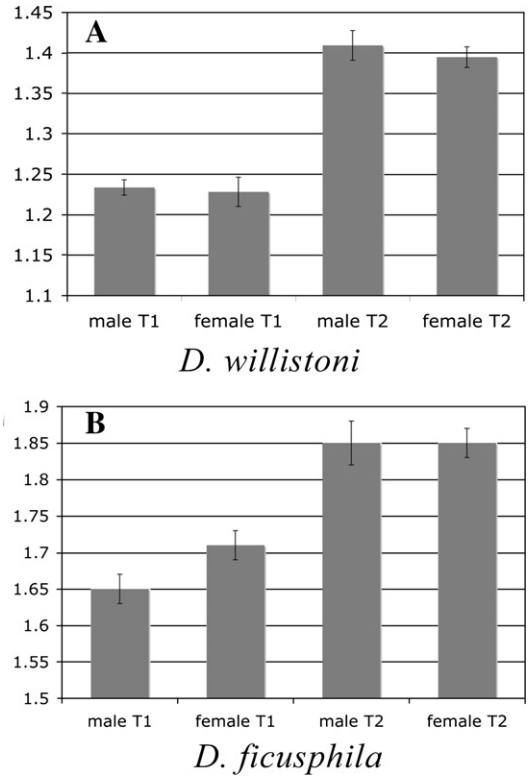

Fig. 5. Differences in *Scr* expression between male and female T1 and T2 legs in *D. willistoni* and *D. ficusphila* measured by quantitative rt-PCR. The *Y* axis shows normalized transcript abundance, estimated as described in Materials and methods. The *Y* scale is reverse exponential so that lower values correspond to higher *Scr* expression. Error bars are standard deviations based on 4 independent biological replicates.

ancestral character states (Pagel et al., 2004). *Scr* expression was recorded as a binary trait (sexually dimorphic or monomorphic) (Fig. 3). We were able to reject the hypothesis that sex-specific *Scr* expression evolved through a single gain followed by multiple losses, as well as the hypothesis that it evolved through multiple independent gains with no losses (Table 1). These hypotheses were rejected for the *melanogaster* species group as a whole, as well as for the Oriental sub-clade (Table 1). Thus, we conclude that sex-specific *Scr* expression has been gained and lost multiple times in the *melanogaster* species group.

All 14 species with rotated sex combs show sex-specific *Scr* expression, while all 11 species that have no sex combs or transverse sex combs have sexually monomorphic *Scr* expression (Fig. 3). We used maximum likelihood and Bayesian reconstruction to quantify this correlation in the phylogenetic framework (Pagel and Meade, 2006). We found that sex comb rotation and sex-specific regulation of *Scr* were highly correlated (Likelihood ratio test, $P<0.0007$; Bayes factor >15.32) (see Materials and methods). This correlation, combined with the experimental analysis of *Scr* function in *D. melanogaster*, strongly suggests that gains and losses of sex-specific regulation of *Scr* have played an important role in the origin and diversification of sex combs.

In some lineages, *Scr* expression has evolved surprisingly rapidly. For example, secondary loss of the sex comb has occurred independently in two species, *D. lucipennis* and *D. prolongata*. The latter species is part of the *rhopaloa* subgroup, whose other members have large, rotated sex combs (Supplementary Fig. 2). *Scr* expression is weak and sexually monomorphic in *D. prolongata* (Fig. 2I), but strongly dimorphic in its closest relatives, *D. rhopaloa* and *KB866* (Figs. 2H and 4). Average coding sequence divergence among these species is only ~1.5%, indicating that the loss of sex-specific *Scr* expression in *D. prolongata* occurred quite recently.

Sex-specific *Scr* expression was gained on an even shorter evolutionary timescale. The *bipectinata* species complex is a monophyletic group of four species — *D. bipectinata*, *D.*

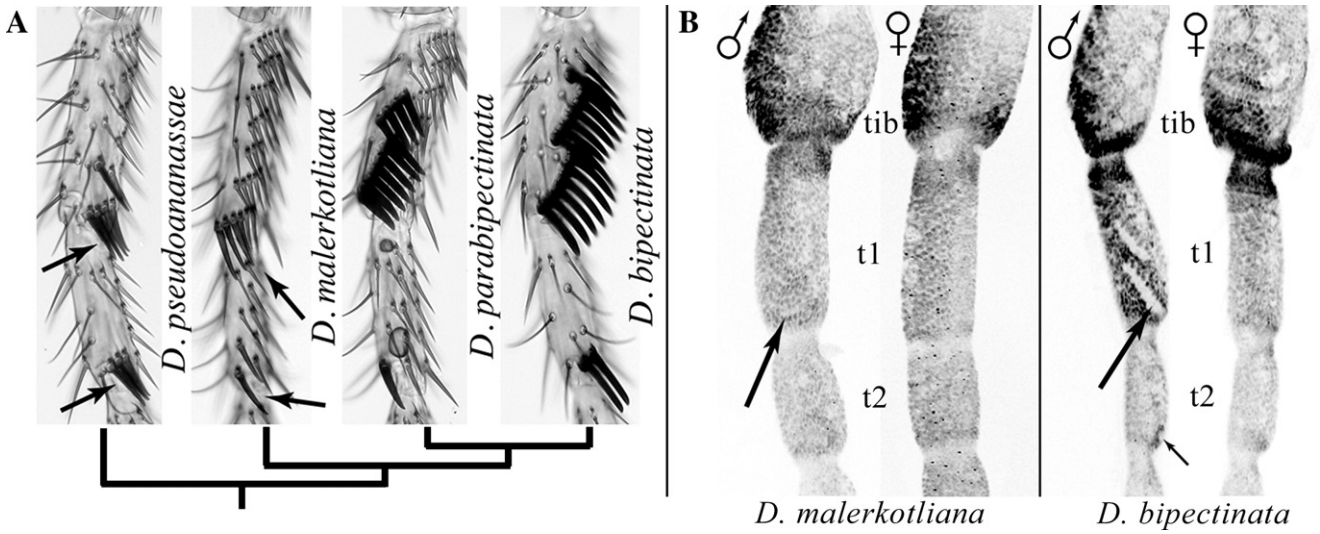

Fig. 6. Recent gain of sex-specific Scr expression in the bipectinata species complex. (A) Sex comb morphology in the four species of the *bipectinata* complex, with phylogenetic relationships indicated below. *D. malerkotliana*, *D. pseudoananassae*, and all 19 other described species of the *ananassae* subgroup have transverse sex combs composed on weakly modified bristles (arrows). *D. bipectinata* and *D. parabipectinata* have evolved rotated sex combs within the last 283,000–385,000 years (Kopp and Barmina, 2005). (B) Pupal *Scr* expression in male and female T1 legs of *D. malerkotliana* and *D. bipectinata*. Note that *Scr* expression is sexually monomorphic in the former species, but dimorphic in the latter. Arrows point to the developing sex combs.

parabipectinata, *D. malerkotliana*, and *D. pseudoananassae* (Bock, 1971; Kopp and Barmina, 2005). *D. bipectinata* and *D. parabipectinata* are unique in the *ananassae* species subgroup in having large, rotated sex combs (Fig. 6). Phylogenetic analysis shows that this is a derived trait in the *bipectinata* complex; the more basal species *D. malerkotliana* and *D. pseudoananassae* have transverse sex combs that are typical of the rest of the *ananassae* subgroup (Fig. 6A and Supplementary Fig. 2). *D. bipectinata*, *D. malerkotliana*, and *D. parabipectinata* are very closely related, having diverged only 283,000–385,000 years ago (Kopp and Barmina, 2005). *D. bipectinata* and *D. malerkotliana* differ in the pattern of *Scr* expression, which is sexually dimorphic in *D. bipectinata* and monomorphic in *D. malerkotliana* (Fig. 6B). Thus, sex-specific regulation of *Scr* must have evolved quite recently in the *bipectinata* complex.

Discussion

HOX genes and the evolution of sex-specific traits

The HOX genes are a perfect illustration of the interplay between conservation and change in evolution. The HOX cluster predates the origin of bilaterally symmetric animals and has an almost universally conserved function of specifying the identity of major regional domains along the anterior–posterior body axis (Carroll, 1995; Carroll et al., 2001; McGinnis and Krumlauf, 1992). At the same time, HOX genes can act as "micromanagers" that control the differentiation of particular morphological structures (Akam, 1998; Crickmore and Mann, 2006; Rozowski and Akam, 2002; Wiellette and McGinnis, 1999). The HOX transcription factors regulate target genes at multiple levels within developmental pathways, including genes involved in terminal cell differentiation (Hersh et al., 2007; Weatherbee et al., 1998). Consistent with this aspect of their function, changes in HOX expression often correlate with the evolution of specific phenotypic traits, such as the morphology of insect mouthparts and crustacean maxillipeds (Averof and Patel, 1997; Mahfooz et al., 2004), hindleg allometry in hemimetabolous insects (Rogers et al., 2002), and the spatial pattern of cuticular projections in *Drosophila* (Stern, 1998).

Sex-specific regulation of *Scr* and its role in generating sex comb diversity give us a new perspective on the interaction between homeotic and sex-determining genes in animal evolution. The joint regulation of target genes by HOX genes and the sex determination pathway plays a key role in the development and evolution of sexually dimorphic structures. For example, the origin of sex-specific abdominal pigmentation in the *D. melanogaster* species group is associated with joint regulation of the *bric a brac* genes by the HOX gene *Abdominal-B* (*Abd-B*) and the sex determination gene *doublesex* (*dsx*) (Kopp et al., 2000). Similarly, *dsx* interacts with *Abd-B* and a variety of signaling pathways to control sexually dimorphic development of the *Drosophila* genitalia (Ahmad and Baker, 2002; Christiansen et al., 2002; Keisman and Baker, 2001; Sanchez et al., 2001). We have now shown that HOX genes themselves can be regulated by the sex determination pathway and that this regulation can evolve rapidly and have an impact on the diversification of secondary sexual traits. An intriguing, though still untested possibility is that *Scr* is a direct target of *dsx* and that evolutionary changes in *Scr* regulation were driven by the gain and loss of Dsx binding sites in its *cis*-regulatory regions.

Gene expression and the reversibility of evolution

There is mounting evidence that morphological evolution is reversible. Secondary loss of sex-specific traits is well documented, including in such charismatic models of sexual selection as peacocks and swordtail fish (Kimball et al., 2001; Wiens, 1999; Wiens, 2001). More intriguing and controversial are instances where, contrary to Dollo's law (Gould, 1970), complex morphological structures have apparently been regained after a previous evolutionary loss. For example, there is strong phylogenetic evidence in favor of secondary re-evolution of wings in stick insects (Whiting et al., 2003), toes in spectacled lizards (Kohlsdorf and Wagner, 2006), coiled shells in limpets (Collin and Cipriani, 2003), and molar teeth in lynx (Werdelin, 1987). Our results raise the possibility that rotated sex combs in *Drosophila* have also been lost and subsequently regained, although the exact sequence of these transitions cannot be reconstructed.

From the standpoint of behavioral ecology, the convergent evolution, secondary loss, and re-evolution of sex-specific traits raise intriguing questions about the co-evolution of male morphology and male and female behavior (Wiens, 2001; Wong and Rosenthal, 2006). Equally important, however, is the developmental-genetic perspective: does convergent evolution and re-evolution of complex morphological structures repeatedly involve the same genes? Unfortunately, few studies to date have addressed this question, although convergent *loss* of traits has been shown to involve the same genes in a number of cases (Colosimo et al., 2004; Cresko et al., 2004; Sucena et al., 2003; Yamamoto et al., 2004). An evolutionary pattern similar to the sex comb is exhibited by another male-specific trait in *Drosophila*, the melanic wing spot, which is also used in mating behavior and has been gained and lost multiple times (Gompel et al., 2005; Kopp and True, 2002; Prud'homme et al., 2006; Yeh et al., 2006). Interestingly, the origin of the wing spot is associated with the gain of a new expression domain by the *yellow* gene in several independent lineages (Prud'homme et al., 2006). We now find that sex-specific expression of *Scr* has also been gained and lost multiple times. Reconstruction of DNA sequence changes responsible for these gains and losses, and identification of other genes that act in parallel with *Scr* to control sex comb morphology, will help elucidate the molecular–genetic mechanisms underlying the convergent evolution and re-evolution of morphological structures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.07.030.

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